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Adaptation to low body temperature influences pulmonary surfactant composition thereby increasing fluidity while maintaining appropriately ordered membrane structure and surface activity

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ABSTRACT

The interfacial surface tension of the lung is regulated by phospholipid-rich pulmonary surfactant films. Small changes in temperature affect surfactant structure and function *in vitro*. We compared the compositional, thermodynamic and functional properties of surfactant from hibernating and summer-active 13-lined ground squirrels (*Ictidomys tridecemlineatus*) with porcine surfactant to understand structure-function relationships in surfactant membranes and films. Hibernating squirrels had more surfactant large aggregates with more fluid monounsaturated molecular species than summer-active animals. The latter had more unsaturated species than porcine surfactant. Cold-adapted surfactant membranes displayed gel-to-fluid transitions at lower phase transition temperatures with reduced enthalpy. Both hibernating and summer-active squirrel surfactants exhibited lower enthalpy than porcine surfactant. LAURDAN fluorescence and DPH anisotropy revealed that surfactant bilayers from both groups of squirrels possessed similar ordered phase characteristics at low temperatures. While ground squirrel surfactants functioned well during dynamic cycling at 3, 25, and 37 °C, porcine surfactant demonstrated poorer activity at 3 °C but was superior at 37 °C. Consequently the surfactant composition of ground squirrels confers a greater thermal flexibility relative to homeothermic mammals, while retaining tight lipid packing at low body temperatures. This may represent the most critical feature contributing to sustained stability of the respiratory interface at low lung volumes. Thus, while less effective than porcine surfactant at 37 °C, summer-active surfactant functions adequately at both 37 °C and 3 °C allowing these animals to enter hibernation. Here further compositional alterations occur which improve function at low temperatures by maintaining adequate stability at low lung volumes and when temperature increases during arousal from hibernation.

1. Introduction

Pulmonary surfactant (PS), a lipo-protein mixture which lines the entire alveolar surface is primarily involved in reducing interfacial surface tension (γ) and thereby lowering the work of breathing and stabilizing the lung [1–5]. In most mammals, pulmonary surfactant comprises ~90% lipids and 10% surfactant proteins (wt/wt) (classified as SP-A, SP-B, SP-C and SP-D) [3,6]. Lipids in PS consist of neutral lipids, especially cholesterol (5–10% of total lipid), but predominantly phospholipids (PL) (~80%) [3,6,7]. Surfactant PLs are characterized by high levels of phosphatidylcholine (PC) (~80%) of which in most mammals >50% are disaturated, including both dipalmitoyl PC (DPPC, also denoted as PC 16:0/16:0) and palmitoyl myristoyl PC (PC 16:0/14:0). Disaturated phosphatidylglycerols (PG) are also present. The remaining PC and PG fractions tend to be enriched in monoenoic species, including palmitoyl palmitoleoyl PC (PC 16:0/16:1) [3,8,9]. DPPC has traditionally been considered the most important surface-active lipid molecule for PS function [8,10]. However, the importance of secondary surfactant phospholipids other than DPPC as

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well as neutral lipids and hydrophobic surfactant proteins in improving surface activity has been demonstrated [11]. Although it is a major component of commercial therapeutic surfactants [12,13], large variations in the content of DPPC/total PC are reported from ~70% in Survanta to 35–55% in most other surfactants including Curosurf, Alveofact, Infasurf and BLES [12,14]. As a result of surfactant lipid composition, pulmonary surfactant membranes exhibit a marked phase transition at temperatures in the physiological range [15,16].

Among mammals, particularly heterotherms that vary their body temperature and marsupials [9], there is considerable species variation in the PL molecular composition of surfactant [17] with DPPC not always being the major PC species. Apparently there is no single PL composition that functions optimally in all mammals. Rather there is a spectrum of molecular compositions presumably with PLs uniquely suited to the physiology of each species [9]. Nor is surfactant lipid composition within individuals static but can change. For example, in heterothermic mammals such as dunnarts (*Sminthopsis crassicaudata*) and bats (*Chalinolobus gouldii*), where body temperature is reduced to low levels during torpor, the composition of whole surfactant may change rapidly due to an increase in cholesterol [18,19]. Interestingly, surfactant from either torpid or warm-active dunnarts or bats lowers surface tension more effectively at *in vitro* temperatures close to the *in vivo* body temperatures of the animals [20,21]. However, it is unclear how the biochemical changes alter membrane thermodynamics or biophysical function under different thermal conditions.

Most investigations on heterothermic mammals have been conducted with small mammals undergoing torpor, which is a short term (i.e. <12 h) decrease in body temperature of ~10–20 °C [9,18,19]. A study on the lung lavage of a reptilian species, the map turtle (*Malaclemys geographica*), which undergoes hibernation for long periods, has shown that unsaturated PC molecules increase at low temperatures [22]. Increased PL unsaturation was also observed in a hibernating mammal, the golden-mantled ground squirrel, where disaturated PC/ total PC falls during hibernation, although the differences reported were not statistically significant [9]. Ground squirrels undergo seasonal hibernation between October and March, where body temperature is maintained at <5 °C [23]. However, hibernating ground squirrels undergo 'arousal', i.e. ~24 h increases in temperature and ventilatory and metabolic rates to summer-active levels every 5–9 days. Here we have chosen another species of hibernating ground squirrel, the 13-lined ground squirrel (*Ictidomys tridecemlineatus)*, to analyze the effects of temperature on the biochemical, thermodynamic and functional properties of warm- and cold-adapted pulmonary surfactant. These properties were compared with the well-characterized natural porcine surfactant from which therapeutic surfactants are derived [12,13]. We hypothesized that the cold-adapted surfactant of hibernating ground squirrels would have a lower phase transition temperature and demonstrate a broader enthalpy of transition than porcine surfactant. Moreover, we hypothesized that cold-adapted surfactant would demonstrate broad functionality at extreme body temperatures.

An improved understanding of surfactant function at physiological extremes may provide insight into surfactant dysfunction and aid in developing ideal exogenous surfactant for therapy in pathological conditions such as respiratory distress, with hypothermic surgical procedures, and in fever.

2. Materials and methods

2.1. Animals

All animal procedures were approved by the University of Western Ontario animal use subcommittee and were in accordance with the Canadian Council of Animal Care's guidelines. 13-lined ground squirrels were wild-caught at the University of Manitoba's field research station in Carmen MB, Canada. After transfer to the University of Western Ontario (London, ON, Canada) animal husbandry procedures described by Muleme et al. [24] were followed as were telemeter implantation and subsequent monitoring of hibernation and sampling. Hibernating ground-squirrels undergo "arousal", ~24 h increases in temperature and respiratory rate to summer-active levels every 5–9 days. Hibernating animals (130.5–154.0 g) were sampled towards the end of the first laboratory hibernation season, 3–4 days into a torpor bout when body temperature was low and constant (~5 °C). Summer-active, non-hibernating squirrels (168.3 g–239.0 g) underwent a hibernation season in the lab but were sacrificed in mid-June when they were actively gaining weight and had a body temperature of ~37 °C. Active animals were euthanized by a sodium pentobarbital overdose (270 mg/ml, 0.2 ml/100 g) whereas hibernating animals were euthanized by cervical dislocation to prevent inducing arousal.

2.2. Lung lavage and surfactant analysis

Immediately following euthanasia PS was collected by repetitive lavage with ice-cold saline through a 14 G angiocatheter secured in the trachea. Briefly, lavage involved slowly instilling 10 ml saline until lungs were fully distended and withdrawing the saline 3 times. A total of 5 lavages were performed and the combined volume was recorded.

The total lavage was centrifuged to remove cells and cellular debris to yield the Total Surfactant (TS) supernatant [25]. Five 1 mL aliquots of this material were frozen at −20 °C for later analysis. The remaining TS was centrifuged to yield the small aggregate (SA) and large aggregate (LA) sub-fractions as previously described [26].

Aliquots of TS, LA and SA subfractions were extracted by chloroform:methanol according to Bligh and Dyer followed by a modified Duck-Chong phosphorus assay in order to analyze the amounts of PL in these samples [27,28]. Cholesterol was measured in LA and SA by an enzymatic colorimetric assay (Wako Chemicals, Richmond, VA) as previously described [25].

PL profiles were determined by electrospray ionization mass spectrometry (ESI-MS). Extracted LA were analyzed by mass spectrometry on a triple quadrupole mass spectrometer (Micromass, Beverly, MA, USA) equipped with a Z-spray source to analyze the PC and PG species, as previously described [26]. Molecular species were identified by their specific molecular weight and relative percentages of each PL species were determined.

Porcine natural surfactant was obtained by lavaging porcine lungs with ice-cold saline and surfactant membranes were purified by density gradient ultracentrifugation as described previously [29,30].

2.3. Differential scanning calorimetry (DSC)

DSC studies were conducted with a Microcal-VP-DSC microcalorimeter (Northampton, MA, USA) as previously described [16], from 5 to 50 °C at a scan rate of 30 °C/h. LA samples were diluted to 0.7–0.9 mg/ml PL with 5 mM TRIS, 150 mM NaCl buffer, pH 7, and this buffer solution was used as a reference. Thermograms and calculations were obtained from 5 entirely reproducible scans of each sample. An experimental baseline, obtained by running buffer in both sample and reference cells, was first subtracted from each thermogram and then the calorimetric parameters described below were calculated using Origin software supplied by Microcal.

We measured enthalpy change (ΔH) as the area under the main thermogram calorimetric peak. ΔH is the energy required for the lipid hydrocarbon chains to overcome intermolecular van der Waals forces during the transition from ordered phases to disordered phases as temperature increases [13,31,32]. We also determined the apparent overall phase transition temperature (T_m), defined as the temperature at which half the total enthalpy of transition is liberated from the sample. The half peak width ($\Delta T½$) reflects the sharpness of the main transition peak and is indicative of the co-operative nature of the transition.

2.4. Fluorescence spectroscopy by LAURDAN

LAURDAN (6-dodecanoyl, 1-2-dimethyl-aminonaphthalene, Invitrogen-Molecular Probes, Eugene, OR) is a fluorescent probe extensively used to study lipid membrane properties in general [33], and properties of surfactant membranes in particular [16,34], due to its sensitivity to environmental polarity. Its emission spectrum is centered around 440 nm in relatively dehydrated, highly packed, ordered membrane phases, while it exhibits a shift in maximum emission to 490 nm in more fluid, hydrated liquid-crystalline membranes [35]. This spectral shift resulting from the temperature-dependent transition of LAURDAN-doped surfactant membranes from ordered to disordered phases was quantified by determination of the so-called generalized polarization function (GP) [36].

LA samples were diluted with 150 mM NaCl, 5 mM TRIS buffer to 0.1 mg/ml PL. LAURDAN in dimethyl sulphoxide was incorporated [15,16], at a molar ratio of 200:1 (lipid: dye). Labeled LA samples were kept at room temperature for 30 min. Finally, the samples were further diluted to 10 μg/ml PL, and transferred to a thermostated cuvette to measure LAURDAN emission intensity (Aminco Bowman series-2 Luminescence spectrofluorimeter) between 10 and 60 °C, corrected by subtracting the baseline intensity values from unlabeled samples and normalized by dividing each value by the highest value at each temperature. Unlabeled samples at similar concentrations were added to a second cuvette to correct for non-specific Raman Effect fluorescence and scattering. The generalized polarization function or GP was calculated at each experimental temperature by the following formula:

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

where I_{440} is the fluorescence intensity at 440 nm and I_{490} is the intensity at 490 nm of the emission spectra. The GP values were plotted against the range of experimental temperatures.

2.5. Fluorescence anisotropy by DPH

The steady-state fluorescence polarization of 1, 6-diphenyl-1,3,5 hexatriene (DPH, Invitrogen Molecular Probes, Eugene, OR) has been used to study the fluidity/order of lipid membranes [37,38]. DPH was added in methanol to aqueous suspensions of surfactant LA samples diluted to 0.5 mg/ml PL, at a molar ratio of 200:1 (lipid: dye) and fluorescence measured between 10 and 60 °C with an Aminco Bowman series-2 Luminescence spectrofluorimeter as described previously [39,40]. Briefly, DPH-doped surfactant LA were excited by linearly polarized light and the DPH emission fluorescence measured through a polarizer situated either parallel or perpendicular to the excitation light [37,41]. The fluorescence anisotropy was calculated by the following formula:

$$r = \frac{I_{II} - I_{\perp}}{I_{II} + 2I_{\perp}}$$

where I_{II} is the fluorescence intensity observed with the excitation and emission polarizers situated in parallel orientation and $I_{\perp}$ is the fluorescence intensity observed with the emission polarizer situated in perpendicular orientation to the polarized light of excitation [41]. Fluorescence anisotropy values were obtained as the mean value after ten measurements at each temperature and plotted against the range of experimental temperatures.

2.6. Surface activity by captive bubble surfactometry (CBS)

Surface activity was examined by a captive bubble surfactometer [42] using a modified protocol [43]. Experiments were conducted at three different temperatures, 3–5 °C, 25 °C and 37 °C. The CBS chamber was filled with nearly 1.5 ml of buffer (5 mM TRIS, 150 mM NaCl, pH 7, 10% sucrose). Duplicate measurements were taken at each temperature for each individual surfactant sample (hibernating squirrel, $n=4$; summer-active squirrel, $n=4$; porcine, $n=3$–5). After degassing the chamber, a small air bubble with approximately 2 mm diameter and 50 μl volume was created in the buffer and floated against the agarose ceiling in the CBS chamber. The outer chamber was filled with distilled water or an ice slurry depending on the temperature required. For experiments at 3–5 °C, ice was continually added to maintain temperature within this range. To conduct experiments at 25 °C and 37 °C, warm or hot air was blown towards the chamber from a blower. Temperature was constantly monitored with a temperature sensor placed in the outer chamber. Samples (~0.3 μl; 20 mg/ml PL) [44,45] were applied to the bubble interface. Bubble shape was recorded using a video camera (Pulnix TM 7 CN; JAI-PULNiX, Glostrup, Denmark) and analyzed using the CBS software to calculate surface tension, area and volume based on the height and diameter of bubbles [43,46].

Following addition of the surfactant, initial adsorption, post-expansion adsorption (PEA) followed by step-wise quasi-static (Q-S, slow) and dynamic (fast, 20 cycles/min) compression–expansion cycles were performed following the procedure described in detail previously [43–47].

2.7. Statistical analysis

All comparisons between hibernating and summer-active squirrels were analyzed with unpaired Student's *t*-tests. For analyses between squirrel and porcine samples, ANOVA was followed by a Student–Newman–Keuls multiple comparison test. Significance was set at $p<0.05$.

3. Results

3.1. Surfactant composition

There was no significant difference in the lavage volumes recovered between summer-active (45.25 ± 3.17 ml) and hibernating (48.33 ± 1.78 ml) ground squirrels. Hibernating animals had significantly more total lavageable surfactant than summer-active animals (Fig. 1). Analysis of the sub-fractions showed that the increase in total surfactant in hibernators was due almost entirely to a 3-fold increase in LA compared with summer-active controls. The SA subfraction did not differ significantly between the two groups. Surfactant pool sizes are traditionally standardized to body mass, but in hibernation the change in body mass is due predominantly to loss of adipose tissue [48], so we expressed the data as the total amount recovered per animal.

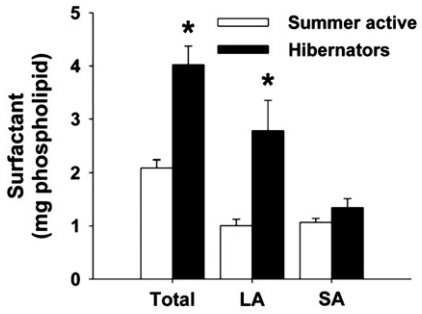

Fig. 1. Surfactant amounts: the amounts of total surfactant in mg phospholipid recovered from the animal, and those of the surfactant subfractions, recovered from the lung lavage of summer-active and hibernating squirrels. Data are expressed as mean ± SE. * indicates a significant difference compared with summer-active animals, $p<0.05$.

Cholesterol content in the LA fraction was similar between hibernators (7.6% of PL (wt/wt) ± 0.5% SEM) and summer-active (7.4 ± 0.6% SEM) animals. SA values were similar (data not shown).

The composition of PC and PG molecular species in LA showed that hibernators had significantly lower amounts of dipalmitoyl PC (DPPC, i.e. PC 16:0/16:0), PC 16:0/14:0, and other disaturated PC species compared with the summer-active animals (total disaturated % PC: 47.6 ± 5.0% in hibernators vs. 65.1 ± 6.6% in summer-active animals; mean ± SEM; $p<0.05$; Fig. 2). These differences were associated with corresponding increases in mono-unsaturated PC species, PC 16:0/16:1 and PC 16:0/18:1. A similar pattern of lower disaturated PG species in hibernators was observed (total disaturated % PG: 22.5 ± 1.1% in hibernators vs 36.2 ± 6.2% in summer-active animals (mean ± SEM; $p<0.05$, Fig. 3).

3.2. Differential scanning calorimetry

Representative DSC thermograms of surfactant from hibernating and summer-active squirrel ($n=3$) and porcine surfactant samples ($n=4$) are shown in Fig. 4 (A–C). Enthalpy (ΔH in calories/mole/°C) associated with the main transition in surfactant from summer-active squirrels (1785.1 ± 58.9) was significantly higher than that from hibernating squirrels (1288.7 ± 48.4; mean ± SEM; $n=3$; $p=0.0029$). Enthalpy associated with porcine surfactant (2847.8 ± 211.0; $n=4$) was significantly higher ($p<0.01$) than for either squirrel group. T_m (°C) of summer-active squirrels (31.6 ± 0.1) and porcine surfactant (32.4 ± 0.3) were both significantly higher ($p<0.001$) than that of hibernating squirrels (29.1 ± 0.2). $\Delta T_{½}$ (°C) was not significantly different among any of the groups (summer-active: 9.2 ± 0.6; hibernating: 10.6 ± 0.6; porcine: 9.4 ± 0.3). The transition onset is relatively lower in hibernating animals (<20 °C) compared with summer-active animals and porcine samples (>20 °C). DSC peaks from all surfactant samples showed marked asymmetry, with a sharp end of transition at high temperature.

3.3. Fluorescence spectroscopy by LAURDAN

Fig. 5A shows LAURDAN mean GP values plotted against experimental temperature for hibernating ($n=3$) and summer-active ($n=3$) ground squirrel samples and porcine samples ($n=4$) doped with this fluorescent probe. The LAURDAN GP curves for both squirrel groups and porcine surfactant are similar at temperatures <20 °C, indicating similar lipid packing. At 30 and 32 °C these values were significantly lower in hibernating squirrel surfactant compared with both summer-active ground squirrel and porcine samples. At 34 °C, GP values of hibernating surfactant samples were significantly lower than for summer-active samples. Above 36 °C there were no differences among the three groups.

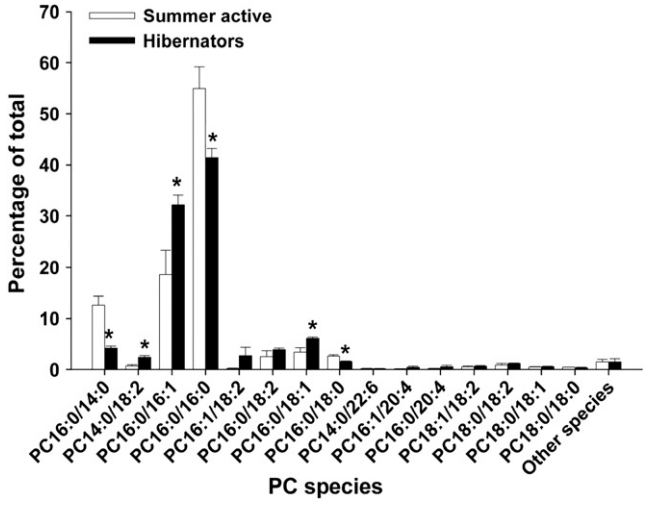

Fig. 2. Surfactant PC composition: the composition, expressed as mole percentage of phosphatidylcholine species in the large aggregate fraction from summer-active and hibernating squirrels. Data are expressed as mean ± SE. * indicates a significant difference compared with summer-active animals, $p<0.05$.

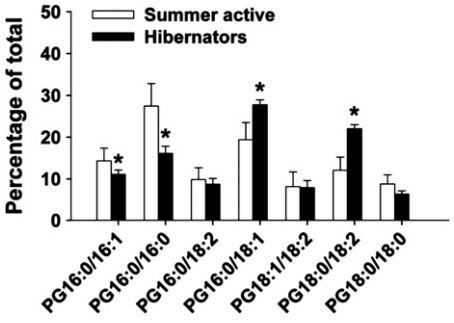

Fig. 3. Surfactant PG composition: the composition, expressed as mole percentage of phosphatidylglycerol species in the large aggregate fraction from summer-active and hibernating squirrels. Data are expressed as mean ± SE. * indicates a significant difference compared with summer-active animals, $p<0.05$.

3.4. Fluorescence anisotropy by DPH

Fluidity of surfactant membrane acyl groups as analyzed by DPH anisotropy suggests a broad ordered-to-disordered transition (Fig. 5B). The average anisotropy values in membrane bilayers from surfactant of hibernating squirrels ($n=3$) were similar to those of summer-active ($n=3$) and porcine samples up to 32 °C. Between 34 and 40 °C, the anisotropy values of summer-active surfactant samples were significantly higher than those of hibernating and porcine surfactant samples. There were no differences above 42 °C.

3.5. Captive bubble surfactometry

Whereas at 25 and 37 °C, initial surfactant adsorption was very fast in all groups (Fig. 6), at 3–5 °C all samples adsorbed substantially

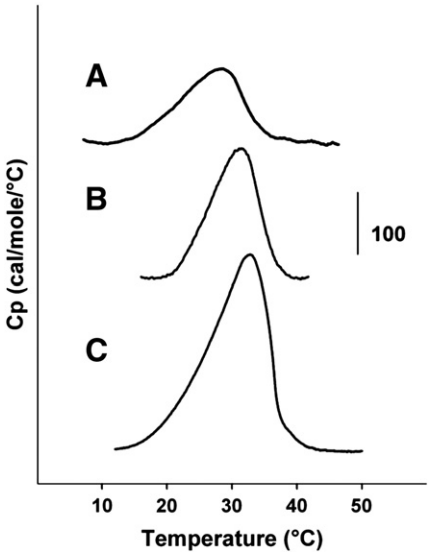

Fig. 4. Differential scanning calorimetry: representative DSC curves of a hibernating (A) and a summer-active squirrel (B) sample and of porcine surfactant (C). Cp is heat capacity or enthalpy expressed in calories/mole/°C. Enthalpy of summer-active squirrels was significantly higher than that of hibernating squirrels. Enthalpy of porcine surfactant was significantly higher than that of both squirrel groups. T_m (°C) of summer-active squirrels and porcine surfactant were both significantly higher than that of hibernating squirrels.

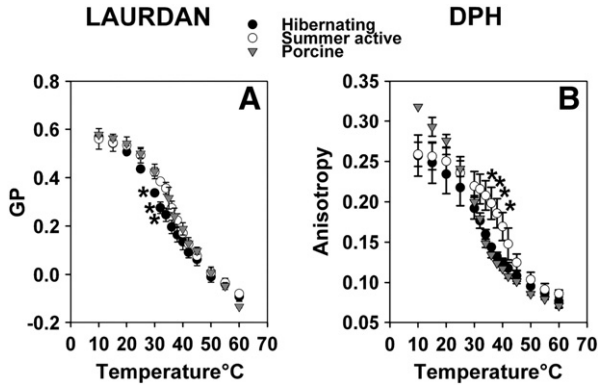

Fig. 5. LAURDAN spectroscopy and DPH anisotropy: thermotropic profiles of normalized LAURDAN generalized polarization (GP) (A) and DPH anisotropy (B) for hibernating (closed circles) and summer-active (open circles) squirrel samples and porcine surfactant (triangles). Data are mean ± SEM after averaging 3 summer-active and 3 hibernating ground squirrel samples. For LAURDAN 4 porcine samples and for DPH 2 porcine samples were averaged. The first data point for porcine DPH anisotropy is from one experiment. * in (A) indicates significantly lower GP values for hibernating samples at 30, 32 and 34 °C. * in (B) indicates significantly higher anisotropy values for summer-active samples compared to those of hibernating and porcine samples at 34, 36, 38 and 40 °C.

slower. At 3–5 °C, both squirrel samples adsorbed substantially slower resulting in significantly higher γ ($p<0.05$) than at 25 or 37 °C. Among the groups, surfactant from hibernating squirrels exhibited the fastest adsorption at low temperature, reaching 25–30 mN/m in 3 to 5 min, while summer-active surfactant reached 30–34 mN/m after 5 min. However, γ_{eq} after 5 min was not significantly different between these samples. Although similar at 25 and 37 °C, porcine surfactant exhibited significantly slower initial adsorption than either squirrel surfactant at 3–5 °C and was unable to attain γ near equilibrium by 5 min (Table 1, Fig. 6).

The PEA technique was designed to reflect respreading of surfactant PL from surfactant reservoirs created during the initial adsorption event. This maneuver reflects events occurring in the lung during the breathing cycle [44]. No differences were observed among the three

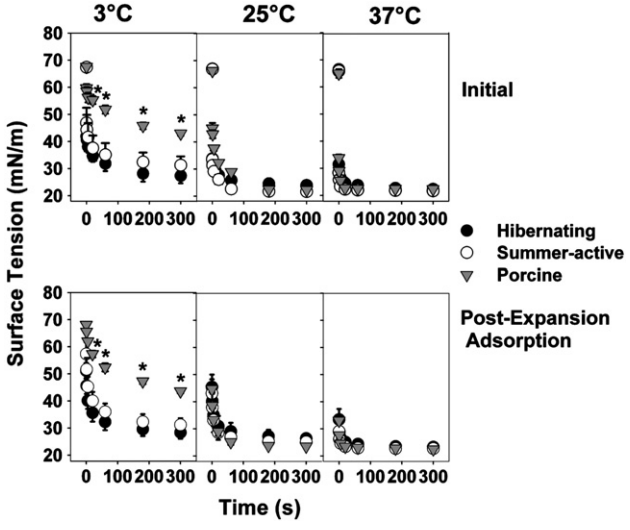

Fig. 6. Initial and post-expansion adsorption: interfacial initial (upper panels) and post-expansion (lower panels) adsorption of hibernating and summer-active squirrel and porcine surfactants, as assessed in the captive bubble surfactometer at different temperatures. Data are mean ± SEM after averaging 4 summer-active, 4 hibernating ground squirrel and 3–5 porcine samples. *indicates a significant difference ($p<0.05$) between porcine surfactant and both summer-active and hibernating ground squirrel surfactant at the time points indicated.

Table 1
Initial and post-expansion adsorption of surfactant from hibernating and summer-active ground squirrels and porcine surfactant determined by captive bubble surfactometry.

Temp	Hibernating		Summer-active		Porcine	
	IA	PEA	IA	PEA	IA	PEA
3 °C	27.4 ± 2.8^{a}	28.4 ± 2.3	31.3 ± 3.2^{a}	31.2 ± 2.5	43.0 ± 0.8^{ac}	43.6 ± 0.7^{ac}
25 °C	24.0 ± 1.5	26.5 ± 1.9	21.7 ± 0.4	25.2 ± 1.7	22.0 ± 0.2	23.5 ± 0.2
37 °C	22.3 ± 0.7	23.3 ± 0.03^{b}	21.7 ± 0.05	22.7 ± 0.1^{b}	22.3 ± 0.4	22.4 ± 0.6

Equilibrium surface tension γ_{eq} (mN/m) after 5 min initial adsorption (IA) and post expansion adsorption (PEA). All data are expressed as mean ± SE.[a] $p<0.05$ compared with γ_{eq} values at 25 and 37 °C; [b] $p<0.05$ compared with γ_{eq} values at 3 and 25 °C; [c] $p<0.05$ compared with γ_{eq} values of both hibernating and summer-active squirrels.

surfactants at 25 or 37 °C, although PEA was somewhat slower at 25 °C (Fig. 6). The curves at 3 °C were similar to those observed during initial adsorption, in that porcine surfactant exhibited delayed γ reduction, significantly higher γ than either squirrel surfactant and was unable to reach equilibrium by 5 min (Table 1 and Fig. 6).

Fig. 7 compares the behavior of ground squirrel and porcine surfactant films subjected to compression–expansion cycling under stepwise (quasi-static, Q-S) or fast (dynamic, 'breathing-like') compression–expansion cycling at different temperatures. Both ground squirrel groups behaved differently between temperatures with statistically significant differences in γ_{min} and area compression (Table 2). However, taken overall surfactant from either squirrel group performed similarly at each temperature. At 3 °C, surfactant of hibernating squirrels reached low γ of ~5 mN/m in the first Q-S cycle with an area compression of ~30%. By cycle 4, area compression required fell to ~13%. The γ_{min} for summer-active surfactant was in the same range but required higher compression, particularly during the first Q-S cycle, where a large plateau was observed (Fig. 7) at ~20 mN/m. Surfactants from both hibernating and summer-active animals showed non-hysteretic isotherms under dynamic compression–expansion cycles at 3 °C.

Surfactants from both squirrel groups showed excellent dynamic isotherms at 25 °C, with low γ_{min} and area compressions and no hysteresis (Fig. 7). Under Q-S cycling, films exhibit isotherms with conspicuous plateaus only in the first cycle. Thereafter, γ_{max} of $\leq$30 mN/m and γ_{min} of $\leq$5 mN/m with only 10–15% area compression were achieved. With fast, 'physiological-like' compression–expansion dynamics, the films maintained their low-compressible state with low stable γ_{max} and γ_{min} values.

At 37 °C, both squirrel materials showed very poor Q-S surface activity, in that they could not produce γ significantly below 20 mN/m upon compression. However, films formed from both hibernating and summer-active surfactants produced low γ when cycled quickly. Thus, ground squirrel surfactant functions relatively well under compression–expansion dynamics over a wide range of temperatures.

In contrast, films from porcine surfactant were particularly inefficient at sustaining compression–expansion dynamics at low temperature, compared to 25 and 37 °C and relative to both hibernating and summer-active ground squirrel surfactant (Table 2; Fig. 7). Isotherms of porcine surfactant at 3 °C also exhibited large hysteresis and substantially higher γ_{max}, probably as a result of poor adsorption and re-spreading activities. Both Q-S and dynamic isotherms of porcine surfactant films demonstrated efficient cycling stabilization at 25 and 37 °C, with significantly lower compression ratios to produce γ near 0 mN/m than squirrel surfactant at 37 °C (Table 2).

4. Discussion

The segregation of ordered and disordered phases in porcine natural and organic extract surfactants is maintained up to ~37 °C (2,15,16). This led to the suggestion that surfactant is poised at the edge of a critical transition (16), which may reflect the fact that PS simultaneously exhibits two apparently contradictory properties.

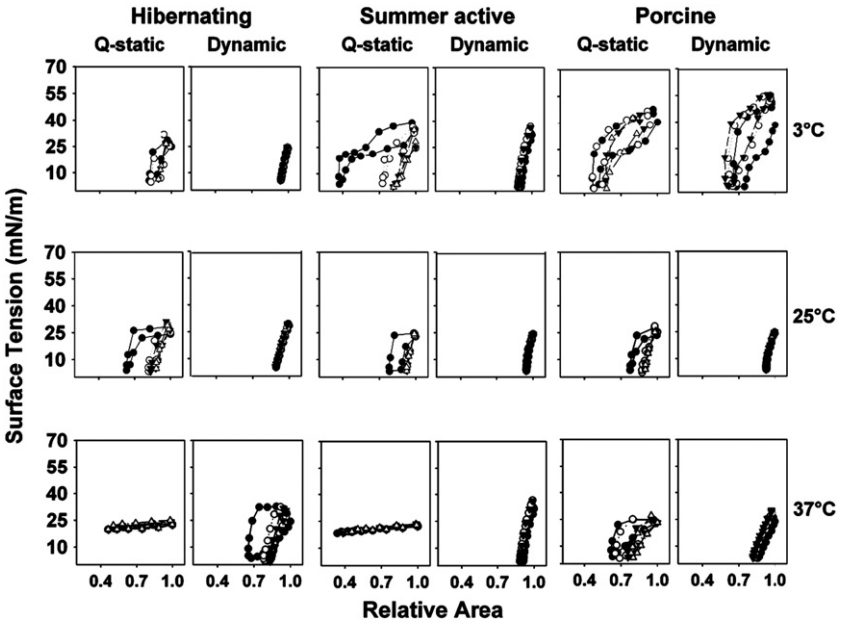

Fig. 7. Quasi-static and dynamic cycles: representative quasi-static and dynamic compression–expansion isotherms for surfactant films from hibernating and summer-active squirrels and porcine samples at different temperatures. Plotted with different symbols are 1st (closed circles), 2nd (open circles), 3rd (closed triangles) and 4th (open triangles) quasi-static and 1st (closed circles), 10th (open circles) and 20th (triangles) dynamic cycles.

Firstly, PS must be sufficiently fluid to adsorb readily as interfacial films and to re-spread during compression–expansion cycling. Secondly, the interfacial, presumably multilayered, film must be able to sustain high surface pressures (low γ) without collapsing at low lung volumes. This is generally considered to depend on the saturated PL species which can sustain very high packing. It has been proposed that PS composition may have evolved as a compromise between the properties of these two states. Recent evidence suggests the disordered regions of surfactant membrane bilayers, which are rich in unsaturated PL and accumulate surfactant proteins, function in surfactant adsorption while the coexisting ordered domains enriched in disaturated PL contribute to the ability to maintain stability under compression [2,5]. This implies that changes in body temperature could be coupled with concomitant adaptive changes in surfactant composition and structure.

It is known that temperature, along with other factors like breathing pattern, alveolar size, diet and developmental stage, induces significant changes to the surfactant system [9,49,50]. Using the model of the 13-lined ground squirrel we here provide biochemical, thermodynamic and biophysical evidence showing how surfactant has adapted while remaining functional over a broad range of temperatures.

Previous studies have shown an increase in total lavageable surfactant in torpid/hibernating mammals [18,19,51]. Here we have further shown that this increase was due to an increase in the LA sub-fraction, which is thought to be the fraction responsible for the functional surface activity of surfactant [52]. This change could be related to the reduction in breathing rate and the change to an episodic breathing pattern observed during hibernation [53].

The PC species of heterothermic mammals that enter short-term torpor are characterized by relatively low (20–45%) DPPC/total PC

Table 2
Surface activity of surfactant from hibernating and summer-active ground squirrels and porcine surfactant determined by captive bubble surfactometry.

	γ_{min} (mN/m) — area compression (%)								γ_{max} (mN/m)	
	Quasi-static cycles				Dynamic cycles					
Hibernating	Q_1	AC_1	Q_4	AC_4	D_1	AC_1	D_{20}	AC_{20}	Q_4	D_{20}
	mN/m	%	mN/m	%	mN/m	%	mN/m	%	mN/m	mN/m
3 °C	5.4 ± 0.3^{a}	33.6 ± 7.2^{a}	5.4 ± 0.8^{a}	13.8 ± 2.0^{a}	5.1 ± 0.6	9.3 ± 1.6^{a}	5.2 ± 0.6	10.3 ± 2.1^{a}	35.2 ± 3.8	36.6 ± 5.2
25 °C	11.7 ± 4.6^{a}	41.4 ± 7.3^{a}	5.1 ± 0.8^{a}	21.3 ± 6.6^{a}	4.2 ± 1.0	15.4 ± 4.3^{a}	4.3 ± 1.0	15.8 ± 4.3^{a}	31.2 ± 2.8	35.3 ± 5.0
37 °C	19.2 ± 0.2	$60.7 \pm 1.0.$	18.1 ± 1.1	53.9 ± 2.4	5.9 ± 1.2	37.1 ± 2.6	9.8 ± 4.3	33.4 ± 6.9	27.0 ± 1.5	42.3 ± 6.1
Summer-active										
3 °C	7.7 ± 2.2^{a}	47.9 ± 11.6	5.4 ± 1.0^{a}	17.2 ± 3.7^{a}	4.9 ± 1.0	11.5 ± 2.7^{a}	5.2 ± 0.9^{a}	13.8 ± 3.6	33.1 ± 3.3^{a}	37.3 ± 4.6
25 °C	4.4 ± 1.0^{a}	31.5 ± 9.0^{a}	4.4 ± 0.5^{a}	10.2 ± 1.6^{a}	4.3 ± 0.3	10.8 ± 3.6^{a}	4.3 ± 0.4	11.6 ± 4.0	27.2 ± 1.4^{a}	28.6 ± 3.3
37 °C	19.6 ± 0.6	58.5 ± 3.0	20 ± 0.4	49.2 ± 3.0	6.3 ± 1.4	36.3 ± 3.1	2.2 ± 0.4	18.8 ± 0.6	23.1 ± 0.2^{d}	32.9 ± 2.2
Porcine										
3 °C	9.5 ± 3.6	52.2 ± 0.7^{b}	5.7 ± 1.1	42.7 ± 0.5^{bc}	4.7 ± 0.5^{a}	35.9 ± 1.5^{bc}	4.4 ± 0.5^{a}	38.1 ± 2.0^{ac}	45.1 ± 1.1^{bc}	54.6 ± 0.2^{bc}
25 °C	4.6 ± 0.3^{a}	17.7 ± 2.6^{d}	2.9 ± 0.8	8.6 ± 0.7	3.7 ± 0.1^{a}	7.0 ± 0.2^{a}	3.7 ± 0.2^{a}	7.4 ± 0.2^{a}	25.2 ± 0.1	24.5 ± 0.4
37 °C	1.2 ± 0.2^{c}	25.3 ± 1.3^{c}	2.0 ± 0.1^{c}	16.4 ± 0.2^{c}	1.9 ± 0.3^{c}	14.0 ± 0.8^{c}	1.9 ± 0.3	14.6 ± 0.4^{c}	25.4 ± 0.8	23.9 ± 3.7

Mean values of minimum surface tension γ_{min} and % area compression (AC) in quasi-static (Q) cycles 1 and 4 (Q_1, AC_1, Q_4, AC_4) and in dynamic cycles (D) 1 & 20 (D_1 AC_1, D_{20} AC_{20}) and maximum surface tension γ_{max} in 4th quasi static cycle Q_4 & 20th dynamic cycle D_{20} of summer-active and hibernating ground squirrels and porcine surfactant. All data are expressed as mean ± SE. [a] $p<0.05$ compared with the corresponding values at 37 °C within the same experimental group. [b] $p<0.05$ compared with corresponding values at 25 and 37 °C within the same experimental group.[c] $p<0.05$ compared with corresponding values at the same temperature of both hibernating and summer-active squirrels. [d] $p=0.05$ compared with corresponding temperature of hibernating animals.

ratios compared with homeothermic mammals (35–60%) including humans [9,54,55]. Our ESI-MS analyses show that surfactant from active squirrels possesses DPPC/total PC levels of ~55% and disaturated PC/total PC levels of ~65%, similar to homeotherms. With hibernation, however, DPPC/total PC fell to ~40% and disaturated PC/total PC to <50%, comparable to the higher levels noted for heterotherms that undergo torpor. Surfactant PL from summer-active squirrels undergoes an increase in monoenoic species in hibernation. For example, PC 16:0/16:1 levels increase progressively from ~15% in adult porcine surfactant [55,56] to ~20% in active and >30% in hibernating squirrels.

Contrasting the pattern in PC noted above, the lack of differences in cholesterol between hibernating and summer-active squirrels differs from some earlier studies with dunnarts and bats which found surfactant cholesterol levels increased in torpor [9,18,19]. Overall, the compositional data suggest that the adaptive strategies may be different for surfactant from mammals experiencing short-term changes in body temperature compared with hibernators that express well-defined seasonal body temperature fluctuations. Short-term thermal changes may not allow for rapid enough adjustment of phospholipid metabolism, and animals may have evolved a compromise surfactant PL composition that may be fine-tuned by modifying cholesterol content. In contrast, hibernators that undergo prolonged decreases in body temperature may have evolved specific PL compositions that function adequately during hibernation.

The combination of increased unsaturated and decreased disaturated PL species in surfactant from hibernators likely accounts for the reduced enthalpies associated with thermotropic ordered-to-disordered membrane phase transitions and the lower T_m measured by DSC. The increase of unsaturated fatty acid chains, which act as spacers, should result in destabilization of ordered phase structures segregating saturated molecules, thereby increasing fluidity and lowering T_m and enthalpy [57]. The presence of asymmetric peaks in surfactant membrane thermograms probably arises from segregated regions or domains with specific lipid composition and packing order/fluidity, which melt at different temperatures. The lipid composition particularly of hibernating squirrel surfactant, would promote a greater level of heterogeneity, resulting in a broader range of packing/order properties, thereby inducing a broader calorimetric transition.

The similar LAURDAN GP profiles (Fig. 5A) and DPH anisotropy (Fig. 5B) for squirrel and porcine surfactants reflect a similar average packing/hydration and ordered state at low temperatures [34], despite differences in composition and thermotropic behavior. However, at intermediate temperatures (32–34 °C for GP and 34–40 °C for anisotropy), physiologically relevant to summer-active ground squirrels, their surfactant possessed a higher order, presumably owing to the higher proportions of disaturated PL. The DPH anisotropy results are complementary to the LAURDAN and DSC results, showing a coexistence of phases in surfactant membranes over a broad range between 10 and 60 °C. Finally, it is evident that despite the higher proportions of unsaturated PL in ground squirrel compared to porcine surfactant, there are sufficient disaturated PL to allow an efficient phase separation, generating a highly-anisotropic ordered phase in both summer-active and hibernating animals at 37 °C.

Captive bubble surfactometry demonstrated that both groups of squirrel surfactant possessed sufficient functional flexibility to adsorb over a wide range of temperatures although surfactant from all animals exhibited faster adsorption at 25 and 37 °C than at 3 °C. It appears that the relatively slower adsorption of surfactant from hibernators at 3 °C is still sufficiently fast to ensure adequate formation and re-spreading of the surface active films during the much slower and episodic breathing pattern of hibernating ground squirrels. Hence, the increased proportion of unsaturated PL in ground squirrel surfactant in general, and in hibernators in particular may be an adaptation to maintain adequate adsorption. The increased levels of LA would also contribute to improved adsorption. In contrast to squirrels, porcine surfactant, with a much lower proportion of unsaturated PL, adsorbed much slower to the interface at 3 °C.

Despite slower adsorption at 3 °C, surfactant from both groups of squirrels exhibited good surface activity during dynamic compression–expansion cycling at all temperatures examined and were able to attain the very low tension required to stabilize the lung. The significantly greater surface area reduction required by porcine surfactant at 3 °C suggests that whereas surfactant from summer-active squirrels could retain adequate function as these animals enter hibernation and during the arousal bouts occurring every 5–9 days, porcine surfactant might not function adequately at such low temperatures.

The relative impairment in surface activity of ground squirrel surfactant under Q-S conditions, particularly at 37 °C (Fig. 7, Table 2), may reflect the fact that summer-active squirrels demonstrate regular, rapid breathing at 37 °C. On the other hand, surfactant from both hibernating and summer-active squirrels demonstrates superior surface activity under Q-S conditions at 3 °C relative to 25 and 37 °C. These results imply simultaneous matched adaptations of surfactant composition to permit heterothermic animals to breathe across a broad spectrum of body temperatures with adaptations in respiratory mechanics to take full advantage of the low respiratory demand to further limit energy expenditure during hibernation. Nevertheless, the surfactant of hibernating squirrels is capable of generating membrane bilayer structures which sustain good interfacial adsorption and spreading to form films which retain sufficient compression-dependent phase separation to remain stable near 0 mN/m surface tension values. This implies that the alterations in the levels of specific saturated and unsaturated molecular species somehow result in a bilayer mixture sufficiently fluid to rapidly transition to films possessing a high enough packing density to generate physical stability under lateral compression at both temperatures. This latter property may be achieved through two specific features: the ability to segregate liquid-ordered DPPC-enriched domains and to form a multilayer-like surface-associated surfactant reservoir which remains associated with the interfacial monolayer [2,4,5].

As stated above, it has long been thought that the unsaturated (fluid phase) PL of pulmonary surfactant facilitates adsorption while the saturated (gel phase) PL components are required for attaining γ near zero during film compression. In this study, we have shown how a surfactant naturally adapted to low body temperatures possesses an increased proportion of unsaturated PL, which confer increased surfactant fluidity enabling phase transition at lower temperature with reduced enthalpy. We speculate that this compositional change may contribute to more efficient melting of the surfactant PL species in hibernating ground squirrels to maintain appropriate fluidity to enable their adsorption to the interface at low temperatures. This is supported by the reduced enthalpy and T_m of surfactant from hibernators compared with summer active animals, while maintaining similar rates of adsorption at low temperature. Nevertheless despite the increased fluidity in cold-adapted surfactant membranes, they demonstrate a similar lipid packing order at low temperature to that seen in summer-active squirrel and in homeothermic porcine surfactants. This characteristic predicates a functional surfactant for both warm- and cold-adapted animals at both high and low temperatures. Captive bubble surfactometry confirmed that both hibernating and summer-active squirrel surfactants remain functional over a wide range of temperatures, whereas porcine surfactant lacks adequate surface activity at low temperatures. We suggest that the higher proportion of specific unsaturated molecular PL species, such as PC16:0/16:1, [49,54,55], may confer a greater flexibility on hibernating species relative to homeothermic mammals. However, further mechanistic and biophysical analyses with model lipid membranes are required to determine the specific molecular mechanisms underlying these adaptations.

5. Conclusion

In conclusion, it therefore appears that evolutionary pressures have resulted in a surfactant composition in summer-active squirrels which, while not optimal, functions sufficiently well to maintain

alveolar stability at 37 °C given the respiration rate of ~250 breaths/min. This particular composition is also sufficiently surface active at 3 °C to allow these animals to enter hibernation. During hibernation, surfactant composition is further modified through increasing unsaturation such that efficiency at low temperatures improves further. Nevertheless functionality at 37 °C is maintained thereby permitting the animals to experience periodic bouts of arousal where temperature returns to 37 °C. The composition and surface properties displayed by the active and hibernating ground squirrels differ greatly from porcine surfactant which although more effective at 37 °C functions poorly and would probably not maintain adequate gaseous exchange at 3 °C.

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